TOXIN GENES FROM THE BACTERIA XENORHABDUS NEMATOPHILUS AND PHOTORHABDUS LUMINESCENS

Field of the Invention:

The present invention concerns the identification and isolation of a new class of protein toxins with specificity for insects, which are produced by bacteria from the genera *Xenorhabdus* and *Photorhabdus*. In addition, the present invention relates to the incorporation of genes encoding this class of toxin into, for example, insect-specific viruses (including entomopox and nuclear polyhedrosis viruses), bacteria (including *Gracilicutes, Firmicutes, Tenericutes* and *Mendosicutes*), yeast and plants for control of insect pests.

Background of the Invention:

Insect pathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* are known to be symbiotically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus* respectively. It has been observed that these bacteria have the ability to kill a wide range of different insects without the aid of their nematode partners. The present inventors have isolated polynucleotide molecules encoding a new class of protein insecticidal toxins from *Xenorhabdus nematophilus* strain A24 and *Photorhabdus luminescens* strain V16/1.

Disclosure of the Invention:

In a first aspect, the present invention provides an isolated polynucleotide molecule encoding an insecticidal toxin, said polynucleotide molecule comprising a nucleotide sequence which substantially corresponds to one of the following;

(i) the nucleotide sequence shown as SEQ ID NO: 1,

(ii) the nucleotide sequence shown as SEQ ID NO: 2, and

(iii) the nucleotide sequence of a portion of (i) or (ii) which encodes an insecticidally-active toxin fragment.

Preferably, said polynucleotide molecule comprises a nucleotide sequence which substantially corresponds to (i) or (ii).

In a second aspect, the present invention provides an isolated polynucleotide molecule encoding an insecticidal toxin, said polynucleotide molecule comprising a nucleotide sequence having at least 85%, more

preferably at least 95%, sequence identity to the nucleotide sequence shown as SEQ ID NO: 2.

In a third aspect, the present invention provides an insecticidal toxin, in a substantially pure form, which comprises an amino acid sequence having at least 70% sequence identity to one of the following:

(i) the amino acid sequence shown as SEQ ID NO: 3,

(ii) the amino acid sequence shown as SEQ ID NO: 4,

(iii) the amino acid sequence of an insecticidally-active toxin fragment of (i) or (ii).

Preferably, said insecticidal toxin comprises an amino acid sequence having at least 85%, more preferably at least 95%, sequence identity to (i) or (ii). Most preferably, the insecticidal toxin comprises an amino acid sequence substantially corresponding to that defined at (i) or (ii).

In a fourth aspect the present invention provides a recombinant microorganism, the recombinant microorganism being characterised in that it is transformed with and expresses the polynucleotide molecule of the first or second aspects of the present invention.

The microorganisms which may be usefully transformed with the polynucleotide molecule of the first or second aspects of the present invention include bacteria, such as *Escherichia, Gracilicutes, Firmicutes, Tenericutes* and *Mendosicutes;* protozoa and yeast. The microorganism can be transformed by routine methods using expression vectors comprising the toxin-encoding polynucleotide molecule operably linked to a suitable inducible or constitutive promoter sequence.

In a fifth aspect, the present invention provides a method of producing an insecticidal toxin, said method comprising:

(i) culturing a microorganism according to the fourth aspect under conditions suitable for the expression of the toxin-encoding polynucleotide molecule, and

(ii) optionally recovering the expressed insecticidal toxin.

In a sixth aspect, the present invention provides a recombinant insect-specific virus, the recombinant insect-specific virus being characterised in that it includes within a non-essential region of its genome the polynucleotide molecule of the first or second aspects of the present invention operably linked to a suitable inducible or constitutive promoter sequence.

The recombinant insect-specific virus of the sixth aspect is preferably selected from entomopox and nuclear polyhedrosis viruses. The recombinant virus can be produced by routine methods such as homologous recombination.

In a seventh aspect, the present invention provides a method for killing pest insects, said method comprising applying to an area infested with said insects an effective amount of a recombinant microorganism according to the fourth aspect and/or a recombinant virus according to the sixth aspect, optionally in admixture with an acceptable agricultural carrier.

In an eighth aspect, the present invention provides a plant transformed with, and capable of expressing, the polynucleotide molecule of the first or second aspects of the present invention.

The plant according to the eighth aspect may be any plant of agricultural, arboricultural, horticultural or ornamental value that is susceptible to damage by feeding pest insects. However, preferably, the plant is selected from plants of agricultural value such as cereals (e.g.; wheat and barley), vegetable plants (e.g.; tomato and potato) and fruit trees (e.g., citrus trees and apples). Other preferred plants include tobacco and cotton.

The plant can be transformed by routine methods including *Agrobacterium* transformation and electroporation. Preferably, the toxin-encoding polynucleotide molecule is operably linked to a suitable inducible or constitutive promoter sequence. Particularly preferred promoter sequences include the cauliflower mosaic virus (CaMV 35 S) promoter element and promoter elements from the sub-clover stunt virus (SCSV).

The term "substantially corresponds" as used herein in relation to the nucleotide sequence is intended to encompass minor variations in the nucleotide sequence which due to degeneracy do not result in a change in the encoded protein. Further this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a.decrease in biological activity of the encoded protein.

The term "substantially corresponding" as used herein in relation to the amino acid sequence is intended to encompass minor variations in the amino acid sequence which do not result in a decrease in biological activity of the insecticidal toxin. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkalamino acids.

As used herein the term "insecticidally-active toxin fragment" is intended to encompass fragments of the insecticidal toxin which retain insecticidal activity as may be determined by, for example, the *Galleria* mellonella bioassay described below.

The term "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

The invention will hereinafter be further described by way of reference to the following, non-limiting example and accompanying figures.

Brief description of the accompanying figures:

Figure 1: Nucleotide sequence of the protein coding (sense) strand of the *X. nematophilus* DNA insert of clone toxb4. The translation initiation codon (ATG) at nucleotide position 17-19 and the translation termination codon (TAA) at nucleotide position 1121-1123 are indicated by shaded boxes. Locations of oligonucleotide sequences used for sequencing primer design are indicated by arrows and a primer name (TOX F1, TOX R3 etc.). Arrows directed left-to-right, positioned above the sequence indicate sense-strand primers, arrows directed right-to-left, positioned below the sequence indicate anti-sense primers.

Figure 2: Deduced sequence of the 368 amino acid toxb4 protein from *X. nematophilus* strain A24, derived by conceptual translation of the long open reading frame commencing at nucleotide position 17 and ending at nucleotide position 1120 of the *toxb4* gene sequence (Fig. 1).

Figure 3: Restriction map of *P. luminescens* V16/1 toxin gene clone showing location of putative toxin protein coding region (solid black box) and direction of transcription (arrow). RI=*Eco*RI, RV=*Eco*RV, H=*Hin*d III, S=*Sma* I. Toxin production from clones containing selected restriction fragments is indicated above the restriction map (+, toxin activity; -, no toxin activity).

Figure 4: Nucleotide sequence of the protein coding (sense) strand of the *P. luminescens Hin*d III/*Sma* I DNA fragment. Translation initiation (ATG) and termination (TGA) codons are indicated by shaded boxes. Locations of oligonucleotide sequences used for sequencing primer design are indicated by arrows and a primer name as described in the brief description of Fig. 1. Restriction enzyme sites used for sub-cloning and identification of sequences necessary for toxin activity are underlined and labelled on the figure.

Figure 5: Deduced sequence of the 335 amino acid PlV16tox1 protein from *P. luminescens* strain V16/1, derived by conceptual translation of the long open reading frame commencing at nucleotide position 172 and ending at nucleotide position 1179 of the *Hin*d III/*Sma* I restriction enzyme fragment (Fig. 4).

Figure 6: Alignment of the nucleotide sequences encompassing the protein open reading frames of the *X. nematophilus* strain A24*toxb4* gene and the *P. luminescens* strain V16/1 *PlV16tox1* gene using the Gap program of the GCG computer software package. The *X. nematophilus* sequence is the upper line and the *P. luminescens* sequence is the lower line.

Figure 7: Alignment of the deduced protein sequences of the extended open reading frames encoding the *X. nematophilus* A24 toxb4 protein and the *P. luminescens* strain V16/1 PlV16tox1 protein using the Gap program of the GCG computer software package. The *X. nematophilus* sequence is the upper line and the *P. luminescens* sequence is the lower line.

Figure 8: Provides a scheme for expressing and isoltating *X.nematophilus* A24toxb4 protein and P.luminescens V16/7 PlV16tox1 protein using the IMPACT™ system. The toxin protein is represented schematically as a solid black bar with the first (Met) and last (Ile) amino acids indicated.

Example 1

Isolation and Characterisation of Toxin genes from *Xenorhabdus nematophilus* A24 and *Photorhabdus luminescens*

Construction of recombinant bacterial DNA libraries

High molecular weight genomic DNA was isolated from *Xenorhabdus nematophilus* strain A24 using the method of Marmur (1961) and from *Photorhabdus luminescens* strain V16/1 by the method of Scott *et al.* (1981). The genomic DNA was partially digested with the restriction enzyme *Sau* 3AI to generate fragments of DNA in the size range 30 to 50 kilobase pairs and dephosphorylated by incubation with the enzyme calf intestinal alkaline phosphatase. The cosmid cloning vector "Supercos" (Stratagene) was linearised by digestion with the restriction enzyme *Bam* HI and ligated to the partially digested bacterial DNA at a vector:genomic DNA ratio of 1:3 according to standard procedures (Maniatis *et al.*, 1982). The ligated DNA was packaged *in vitro* using Gigapack II XL Packaging Extract according to manufacturer's instructions (Stratagene). The packaged DNA was transfected into the *Escherichia coli* strain NM554 (F', *rec*A, *ara*D139, Δ(*ara, leu*) 7696, Δ*lac* Y74, *gal*U-, *gal*K-, *hsr*, *hsm*$^+$, *str*A, *mcr*A[-], *mcr*A[-]). Transfected bacteria were plated onto Luria Bertani (LB) agar medium containing 150μg ml^{-1} ampicillin, to select for bacteria containing recombinant cosmid clones.

Isolation of an insect toxin gene from Xenorhabdus nematophilus strain A24 by functional screening

Cultures of bacteria harbouring individual cosmid clones were grown overnight at 28°C in LB broth containing 150μg ml^{-1} ampicillin. The bacterial cultures were treated for 15 minutes with 2mg ml^{-1} lysozyme to create cell-free lysates. Five microlitre aliquots of these lysates were injected into the haemocoel of three *Galleria mellonella* fourth instar larvae. Two clones with insecticidal activity were identified. Control lysates prepared by lysozyme treatment of *E. coli* NM554 cells containing non-recombinant Supercos vector possessed no toxin activity in the *Galleria* bioassay.

Characterisation of Toxin Producing Clones

Cosmid DNA from toxin-expressing clones was isolated using a standard alkaline lysis procedure (Maniatis *et al.*, 1982). Isolated DNA was analysed by restriction enzyme digestion and agarose gel electrophoresis (Maniatis *et al.*, 1982). Both cosmid clones appeared to contain the same

region of approximately 34.6 kb of *X. nematophilus* genomic DNA. One clone, designated cos149 was chosen for further analysis.

A 7.4 kb *Bam* HI fragment from cos149 was ligated into the plasmid vector pGEM7Z(f)+ (Promega Biotec) and transformed into the *E. coli* strain DH5a (F', F80dlac ZΔ M15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 [r_{K-} m_{K+}] *sup*E44, *rel*A1, *deo*R, Δ[*lac*ZYA-*arg*F] U169) using electroporation at 25mF, 200 and 2.5kV in a 0.2cm cuvette in a Bio-Rad Gene Pulser. The resultant sub-clone was designated N8pGEM. Lysates prepared from *E. coli* cells containing the N8pGEM clone contained toxin as determined by the *Galleria* haemolymph injection bioassay.

A set of unidirectional deletion clones was prepared from N8pGEM according to the method of Henikoff (1984) using the Erase-a-base kit (Promega Biotec) and digestion with the enzymes *Cla* I and *Sph* I. Deleted DNA was recircularised by ligation with T4 DNA ligase and transformed into *E. coli* strain DH5α by electroporation as described above. Deletion sub-clones of varying sizes were identified and tested for toxin production using the *Galleria* bioassay. The smallest clone that retained toxin expression (designated tox 1) contained 1.5kb of *X. nematophilus* DNA.

Plasmid DNA from the tox 1 clone was isolated, digested with the restriction enzymes *Sac* I and *Hin*d III and directionally deleted with the Erase-a-base kit. A set of deleted clones was identified and tested for toxin production. The smallest clone retaining toxin activity (designated toxb4) contained 1.2kb of *X. nematophilus* DNA. The toxb4 clone was sequenced on both strands with a combination of vector and gene-specific sequencing primers and ABI Prism™ di-deoxy dye-terminator sequencing mix (Applied Biosystems). Plasmid DNA was prepared by a standard alkaline lysis procedure (Maniatis *et al.*, 1982), the double-stranded DNA was sequenced by a thermal cycle sequencing protocol, and sequencing reactions were analysed on an automated DNA sequencer (Applied Biosystems Model 377) according to manufacturer's instructions.

The toxb4 clone contained an insert 1205bp in length (Figure 1) which encoded a protein open reading frame of 368 amino acid residues (Figure 2). Searches of the non-redundant Genbank nucleotide and protein databases were done for the toxb4 nucleotide and deduced protein sequences using the blastn, fasta and blastp, programs for DNA and protein sequences. No

statistically significant similarity was detected between the *X. nematophilus* sequences and sequences present in the databases.

Isolation of a toxb4 homologue from Photorhabdus luminescens strain V16/1

The genomic DNA cosmid library prepared from *P. luminescens* strain V16/1 was screened by nucleic acid hybridisation using the *toxb4* gene as a hybridisation probe. Two hundred clones were grown overnight at 37°C on LB agar plates containing 150μg ml^{-1} ampicillin and the resultant bacterial clones were transferred to nylon membrane discs (Colony/Plaque Screen™, NEN DuPont) according to the manufacturer's protocol. Colonies were lysed in situ on the membranes by treatment with 0.5 N NaOH and neutralised with 1.0M Tris-Cl, pH 7.5, and the cosmid DNA was immobilised on the membranes by air drying. Filters were pre-hybridised in a solution consisting of 5X SSPE, 0.2% w/v skim-milk powder, 0.5% w/v SDS and 0.2% mg/ml denatured salmon sperm DNA at 68°C for 3 hours. A hybridisation probe was prepared by radiolabelling approximately 100ng of isolated *toxb4* DNA with 50 μCi α-^{32}P-dATP by random-primed synthesis using the Gigaprime DNA labelling kit (GPK-1, Bresatec). Filters were incubated with the *toxb4* probe in 5X SSPE, 0.2% w/v skim-milk powder, 0.5% w/v SDS and 0.2% mg/ml denatured salmon sperm DNA at 68°C overnight. Filters were rinsed briefly in 2X SSC, and washed once for 15 min at room temperature in 2X SSC, 0.1% w/v SDS, once at 68°C for 30 min in 0.5X SSC, 0.2% SDS. After a final rinse in 0.5X SSC filters were autoradiographed for 24 hours at -80°C. Three clones that hybridised with the *toxb4* probe were identified. Cultures were grown for each clone and cell lysates were assayed for toxicity using the *Galleria* bioassay. Two clones, designated cos154 and cos160 showed toxin expression. Cosmid DNA was isolated from cos154 and cos160 and analysed by restriction enzyme digestion and Southern blot hybridisation. An 8.5kb *Not* I restriction enzyme fragment that hybridised to the *toxb4* probe was isolated from clone cos160 and sub-cloned into the *Not* I site of the plasmid vector pBC (KS)+ (Stratagene). Further restriction enzyme mapping and bioassay resulted in identification of a 2.4 kb *Eco* RI fragment that contained all the sequences necessary for production of active toxin.

Characterisation of the P. luminescens strain V16/1 toxin gene

Three additional sub-clones of the 2.4 kb *Eco* RI fragment were constructed and tested for toxin production (Figure 3). A 1.65kb *Hin*d III/*Eco* RI fragment, a 1.39kb *Hin*d III/*Sma* I fragment and a 1.44kb *Eco* RV/*Eco* RI

fragment were each ligated into the plasmid vector pBluescript II (KS)+ (Stratagene) and the ligated DNA was transformed into *E. coli* strain DH10B™ (Stratagene) (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) F80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G). Cell lysates were prepared from cultures containing each of these sub-clones and bioassayed by haemocoel injection into *Galleria* larvae. Cultures containing the 1.65kb *Hin*d III/*Eco* RI fragment and the 1.39kb *Hin*d III/*Sma* I fragment expressed active toxin but cultures containing the 1.44kb *Eco* RV/*Eco* RI fragment were inactive in the bioassay (Fig. 3). Thus, sequences located 5' to the *Eco* RV site of the *P. luminescens* V16/1 *Hin*d III/*Eco* RI fragment are required for toxin expression from the plasmid pBluescript II (KS)+, whereas sequences 3' to the *Sma* I site are dispensable. The toxin gene is designated *PlV16tox1* and the toxin protein encoded by this gene is designated PlV16tox1. A strategy was developed for sequencing the 1.39 kb *Hin*d III/*Sma* I *P. luminescens* DNA fragment based on internal restriction enzyme sites and custom-synthesised oligonucleotide sequencing primers. The complete sequence of the 1.39 kb *Hin*d III/*Sma* I fragment was determined on both strands (Figure 4). Analysis of this DNA sequence identified a single long open reading frame 335 amino acid residues in length (Figure 5).

Comparison of the toxin gene and protein sequences from X. nematophilus and P. luminescens

The DNA sequences corresponding to the deduced toxin protein open reading frames were compared for the two bacterial species using the 'Gap' program of the GCG software package. The two gene sequences are 83% identical in the coding region (Figure 6) but show no significant similarity in the sequences immediately 5' and 3' of the extended open reading frame. The toxin protein sequences were likewise compared with the 'Gap' program and found to be 75% identical to each other and 86% similar if physico-chemically conservative amino acid differences were taken into consideration (Figure 7). The existence of two extended insertion/deletion variants between the two proteins identifies amino acids that are not essential for toxic activity against *Galleria melonella*.

Example 2:

Distribution of the Toxin gene from *X.nematophilus* A24

Genomic DNA was prepared from the type strain for each of four identified *Xenorhabdus* species, an additional unclassified *Xenorhabdus* species and six *Photorhabdus luminescens* strains selected to include at least one member of each of the major genetic groups identified by analysis of 16S ribosomal RNA genes (Brunel *et al.*, 1997). The DNA was digested with restriction enzymes, fractionated by agarose gel electrophoresis and transferred to nylon membranes by the Southern blot method (Maniatis *et al.*, 1982) The filters were hybridised with a probe prepared from the *X. nematophilus* A24 toxb4 gene. Hybridisation conditions were selected that would allow sequences with an average identity of approximately 65% to be detected. The results are shown in Table 1.

Table 1

Bacterial species	Strain	Toxin gene[†]
Xenorhabdus nematophilus	A24	+
Xenorhabdus nematophilus	AN6	+
Xenorhabdus poinarii	G6	-
Xenorhabdus beddingii	Q58	-
Xenorhabdus bovienii	T28	-
Xenorhabdus sp.	K77	-
Photorhabdus luminescens	Hb	-
Photorhabdus luminescens	Hm	-
Photorhabdus luminescens	C1	-
Photorhabdus luminescens	V16	+
Photorhabdus luminescens	C8406	+
Photorhabdus luminescens	K81	+

[†] + indicates presence of hybridising DNA, - indicates absence of hybridisation of toxin gene probe.

Clearly, homologues of the toxin gene from *X.nematophilus* A24 is present in some species of the genus *Xenorhabdus*, and some, but not all isolates of *Photorhabdus luminescens*.

Example 3:

Activity of Toxin genes cloned into plasmid vectors and transformed into *E. coli*

Active toxin protein was expressed when the A24 toxb4 clone or V16 tox1 genes were inserted into general plasmid vectors of the type pGEM (Promega Biotec) or pBluescript (Stratagene) and the recombinant plasmids transformed into *E. coli*. More specifically, the *X. nematophilus* toxin A24 toxb4gene was cloned into the plasmid pGEM7z and the *P. luminescens* V16 tox1 gene was cloned into pBluescript SK.

Preparation of cell extract

A culture of *E. coli* cells transformed with either a recombinant plasmid containing a toxin gene or a non-recombinant parent plasmid was grown overnight at 37°C in nutrient broth. Lysozyme was added to the culture to a final concentration of 1 mg/ml and the mixture left at room temperature for 30 minutes to lyse the cells. The cleared lysate was used directly for bioassay.

Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Ten microlitres of *E. coli* cell lysate were injected into the abdominal region of a *Galleria mellonella* larvae through an intersegmental membrane. Bioassays were done on 10 larvae for each extract and injected larvae were held at 22°C. Mortality was recorded daily. Results are shown in Table 2.

Table 2

Toxin source	Percentage mortality				
	Day 1	Day 2	Day 3	Day 4	Day 5
PlV16tox1	0	20	40	90	100
pBluescript SK (control)	0	10	10	10	20
A24toxb4	10	10	10	100	100
pGEM7z (control)	0	0	10	20	20

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P luminescens* strain V16/1 kill *G. mellonella* larvae and caused complete mortality of injected individuals five days after injection. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z did not kill the larvae.

Effect of Temperature on Toxicity

Extracts were prepared from *E. coli* cells transformed either with cloned toxin genes or the empty plasmid vector controls and injected into *G. mellonella* larvae as described previously. The injected larvae were maintained at either 20°C or 25°C. Results are shown in Table 3.

Table 3

Toxin source	Temp	Percentage mortality					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
PlV16tox1	20°C	0	10	25	60	90	100
PlV16tox1	25°C	0	0	100	100	100	100
pBluescript SK	20°C	0	0	0	0	0	0
pBluescript SK	25°C	0	5	10	10	15	15
A24toxb4	20°C	5	30	35	65	95	100
A24toxb4	25°C	60	75	100	100	100	100
pGEM7z	20°C	0	5	5	5	5	5
pGEM7z	25°C	0	5	5	5	5	5

Extracts prepared from cells containing either the cloned toxin gene from *X. nematophilus* A24 or the *P. luminescens* V16 toxin gene killed all larvae within three days for larvae held at 25°C or by six days for larvae maintained at 20°C following injection. Control extracts prepared from cells containing only the cloning vectors pBluescript or pGEM7z did not cause significant larval mortality.

Example 4:

Toxin Activity Against Different Insect Species

(1) Helicoverpa armigera (Lepidoptera:Noctuidae)

Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Ten microlitres of *E. coli* cell lysate were injected into the abdominal region of fourth instar *Helicoverpa armigera* larvae through an intersegmental membrane. Bioassays were done on 24 larvae for each extract and injected animals were held at 27°C. Mortality was recorded daily. Results are shown in Table 4.

Table 4

Toxin source	Percentage mortality			
	Day 1	Day 2	Day 3	Day 4
PlV16tox1	38	71	87	91
pBluescript SK	4	4	8	8
A24toxb4	50	87	91	91
pGEM7z	0	0	0	0

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P luminescens* strain V16/1 caused significant mortality to injected larvae within 24 hours after injection. All larvae died by 4 days following the injection, with the exception of a small number of "escapees" that resulted from leakage of injected material upon removal of the injection needle. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z had no significant effect on *H. armigera* larvae.

(2) Plodia interpunctella (Lepidoptera:)

Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Five microlitres of *E. coli* cell lysate were injected into the abdominal region of a final instar *Plodia interpunctella* larva through an intersegmental membrane. Bioassays were done on 20 wandering-stage larvae for each

extract and injected animals were held at 26°C. Mortality was recorded daily. Results are shown in Table 5.

Table 5

Toxin source	Percentage mortality		
	Day 1	Day 2	Day 3
PIV16tox1	20	90	100
pBluescript SK	0	0	0
A24toxb4	75	95	100
pGEM7z	0	5	5

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P luminescens* strain V16/1 caused significant mortality to injected larvae within 24 hours after injection. All larvae had died within 3 days. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z had no significant effect on survival of *P. interpunctella* larvae.

(3) Lucilia cuprina (Diptera:Calliphoridae) Adults Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Five microlitres of *E. coli* cell lysate were injected into the abdomen of a 3 day old *Lucilia cuprina* female fly through an intersegmental membrane. Bioassays were done on 20 flies for each extract and injected animals were held at 25°C. Mortality was recorded daily. Results are shown in Table 6.

Table 6

Toxin source	Percentage mortality			
	Day 1	Day 2	Day 3	Day 4
PIV16tox1	55	65	85	100
pBluescript SK	20	25	25	25
A24toxb4	55	75	85	100
pGEM7z	30	60	65	65

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P luminescens* strain V16/1 caused significant mortality to injected flies within 24 hours of injection. All flies died by 4 days after injection. Extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z also caused significant mortality to the *L. cuprina* flies in the first 48 hours following injection. After this control mortality stabilised, there was no further deaths for the remainder of the test period. Additional experiments with saline injections showed that the early mortality in the control group resulted from physical damage to the flies as a result of the injection process.

(4) Lucilia cuprina (Diptera:Calliphoridae) Larvae Bioassay

Extracts were bioassayed using the intrahaemocoel injection assay. Five microlitres of *E. coli* cell lysate were injected into the abdominal cavity of wandering-stage final instar *Lucilia cuprina* larvae through an intersegmental membrane. Bioassays were done on 20 larvae for each extract and injected animals were held at 25°C. Mortality was recorded daily. Results are shown in Table 7.

Table 7

Toxin source	Percentage mortality			
	Day 1	Day 2	Day 3	Day 4
PlV16tox1	35	45	75	80
pBluescript SK	25	30	30	30
A24toxb4	10	35	90	95
pGEM7z	15	20	20	25

Extracts prepared from *E. coli* cells transformed with recombinant plasmids containing the toxin gene from either *X. nematophilus* A24 or *P luminescens* strain V16/1 caused significant mortality to injected larvae within 48 hours of injection. All larvae died by 4 days after injection, with the exception of a small number of "escapees" resulting from leakage at the time of needle withdrawal as previously described for *H. armigera*. As with

the *L. cuprina* adults, extracts prepared from cells containing only the plasmid vectors pBluescript SK or pGEM7z caused significant mortality to the *L. cuprina* larvae in the first 48 hours following injection. After this, control mortality stabilised and there were no further deaths in this group of larvae for the remainder of the test period. As described above, experiments with saline injections showed that this early mortality in the control group resulted from physical damage to the larvae as a result of the injection process.

(5) Aphis gossypii (Hemiptera:Aphididae) Nymphs Bioassay

Extracts were prepared from *E. coli* cells containing either the *X. nematophilus* toxin gene or the empty plasmid vector pGEM7z. The extracts were incorporated into a defined liquid diet at a concentration of 10% by volume and aphids were provided *ad libitum* access to diet for a period of five days. Results are shown in Table 8.

Table 8

Treatment	% Mortality at day 5	Average Number of Moults
Control[†]	10	1.9
pGEM7z extract	0	2
A24toxb4 extract	90	0.6

[†] an additional treatment consisting of diet supplemented with lysozyme at the same final concentration used to prepare the *E. coli* cell extracts was included as a control for any potential effects of the lysozyme.

The *X. nematophilus* A24 toxin effectively blocked growth as seen from the reduction in the number of nymphal moults, and by five days had killed most of the larvae. Thus, the *X. nematophilus* A24 toxin was orally insecticidal to *Aphis gossypii*.

Example 5:

Expression and Purification of the Full-length Toxin Protein from *X.nematophilus*

Further characterisation of the properties of the toxins encoded by the cloned genes from *X. nematophilus* A24 and *P. luminescens* V16/1 required expression of the full-length protein in a format that allowed for affinity purification of the toxin. This was achieved by expressing the full-length toxin as a fusion protein in which the fusion partner was used for affinity selection, and the toxin domain was cleaved off chemically after the purification stage. A suitable expression and purification system is the IMPACT™ system (New England Biolabs) in which the toxin open reading frame is cloned at the 5' end of a self-splicing intein coding sequence fused to a short DNA sequence encoding a chitin binding domain.

Recombinant plasmids containing both the *X. nematophilus* A24 toxin and the *P. luminescens* V16/1 toxin genes were prepared in the IMPACT™ vector pCYB3 (Figure 8). Preparation of these constructs required the engineering of a unique restriction enzyme site at each end of the toxin open reading frame that enabled in-frame insertion of the toxin gene into the expression vector such that translation began at the Methionine initiation codon of the toxin protein and a cleavage site for protein splicing was placed immediately adjacent to the final residue of the toxin open reading frame. Expression of the fusion proteins in *E. coli*, preparation of bacterial cell extracts, affinity isolaton of the fusion proteins on chitin cellulose columns, on-column DTT-mediated cleavage of the fusion proteins and elution of the purified toxin proteins were all performed according to the manufacturer's instructions (IMPACT™ system manual, New England Biolabs)

For both toxin constructs a major protein product of the expected size (approximately 40 kDa) was detected by SDS polyacrylamide gel electrophoretic analysis of the column eluate. The preparations contained several other proteins but these comprised less than 10% of the total protein present in the samples as determined by Coomassie blue staining of the polyacrylamide gels. Approximately 750µg of PlV16tox1 toxin and 1.5mg of A24toxb4 toxin were isolated from one litre of *E. coli* broth cultures. Purified proteins were dialysed against phosphate-buffered saline and simultaneously concentrated by diafiltration to a final concentration of approximately

1mg/ml on Millipore spin cartridges with a membrane nominal molecular weight cut-off of 10kDa according to manufacturer's instructions (Millipore).

Example 6:

Biological Activity of Purified Toxin Proteins

Bioassay

The activity of the purified *X. nematophilus* and *P. luminescens* toxins were determined by intra-haemocoel injection bioassay on *Galleria mellonella* and *Helicoverpa armigera* larvae as described above. The toxin protein preparations were diluted in phosphate-buffered saline and 10ml of protein solution was injected into each larva. Ten larvae were injected for each protein concentration and mortality was recorded at 12 hour intervals for six days after injection. Proteins were tested over a dose range from 1 nanogram (10^{-9}g) to 1 microgram (10^{-6}g) of protein per larva. An inert protein, *E. coli* maltose binding protein, was prepared in the IMPACT™ system, purified and concentrated according to the same methods used for the two toxin proteins. The purified maltose binding protein was used as a control for these experiments. The maltose binding protein did not cause larval mortality at any of the quantities tested. The results are shown in Tables 9 to 12.

Table 9:

Effect of purified PlV16 tox1 toxin on *G. mellonella* larvae

Protein Injected	Percentage Mortality								
	Day2 am	Day2 pm	Day3 am	Day3 pm	Day4 am	Day4 pm	Day5 am	Day5 pm	Day6 am
1ng	0	0	0	0	0	0	0	0	0
10ng	0	0	0	0	0	0	0	0	10
20ng	0	10	10	20	20	20	20	30	30
100ng	0	0	30	40	60	70	80	80	100
200ng	0	0	44	56	56	78	100	100	100
1000ng	20	20	60	60	100	100	100	100	100

Table 10:

Effect of purified A24 toxb4 toxin on *G. mellonella* larvae

Protein Injected	Percentage Mortality								
	Day2 am	Day2 pm	Day3 am	Day3 pm	Day4 am	Day4 pm	Day5 am	Day5 pm	Day6 am
1ng	0	0	0	0	0	0	0	0	0
10ng	0	0	0	0	0	0	0	0	0
20ng	0	0	10	20	20	40	60	70	80
100ng	10	10	20	30	30	50	90	100	100
200ng	0	0	0	0	50	70	70	90	100
1000ng	0	0	0	10	60	80	100	100	100

Table 11:

Effect of purified PlV16 tox1 toxin on *H. armigera* larvae

Protein Injected	Percentage Mortality							
	Day1/am	Day1/pm	Day2/am	Day2/pm	Day3/am	Day3/pm	Day4/am	Day4/pm
1ng	0	0	0	0	0	0	0	0
10ng	0	0	0	0	0	0	0	0
20ng	0	10	10	10	10	10	10	10
100ng	30	30	50	50	60	70	70	70
200ng	0	0	80	80	80	80	80	80
1000ng	22	67	100	100	100	100	100	100

Table 12:

Effect of purified A24 toxb4 toxin on *H. armigera* larvae

Protein Injected	Percentage Mortality							
	Day1/am	Day1/pm	Day2/am	Day2/pm	Day3/am	Day3/pm	Day4/am	Day4/pm
1ng	0	0	0	0	0	0	0	0
10ng	0	30	50	70	90	90	90	90
20ng	0	30	50	80	90	90	90	90
100ng	0	20	80	100	100	100	100	100
200ng	0	30	90	100	100	100	100	100
1000ng	20	60	100	100	100	100	100	100

Both the *X. nematophilus* A24 toxin and the *P. luminescens* V16/1 toxin killed a high percentage of larvae after a single injection of at least 20ng of toxin protein per larva. Mortality was dependent on toxin type and concentration. *H. armigera* was sensitive to small quantities of *X. nematophilus* A24 toxin with high mortality at 10-20ng of toxin per larva, but was less sensitive to *P. luminescens* V16/1 toxin where significant mortality was observed only for quantities greater than 20ng of protein per larva. A similar pattern of sensitivity was observed for *G. mellonella* larvae. The time taken to kill the larvae of either species was not strongly dependent on the time since toxin injection, although larger amounts of toxin killed more quickly. However, at all quantities greater than, or equal to 20ng per larva the insects were effectively dead, because the *H. armigera* larvae ceased feeding and *G. mellonella* larvae were unable to spin cocoon silk.

Thus, the proteins encoded by the A24 toxb4 genes of *X. nematophilus* and the PlV16 tox1 gene of *P. luminescens* encode toxin proteins that are effective insecticides, especially of lepidopterous larvae including *G. mellonella, H. armigera* and *P. interpunctella,* when delivered into insect haemocoel.

Example 7:

Effect of Purified Toxin on Insect Cells in Culture

The purified *X nematophilus* A24 toxin and *P. luminescens* V16/1 toxin and the maltose binding protein control were each tested for their effects on the growth and viability of insect cells in tissue culture. A sample of 10^4 cells in the appropriate culture medium was mixed with the test proteins at several different concentrations and seeded into the wells of a 96-well tissue culture plate. Cells were allowed to grow for 24 hours at 25°C and cells were counted in a haemocytometer and assessed visually for cell lysis. The results are shown in Table 13.

Table 13

Cell Line	Treatment		Cells/well
	Toxin	Concentration µg/ml	
Schneider 2	PlV16tox1	0	$4.1x10^4$
	"	0.001	ND†
	"	0.1	$4.1x10^4$
	"	1	$4.6x10^4$
Schneider 2	A24toxb4	0	$3.7x10^4$
	"	0.001	ND
	"	0.1	$3.6x10^4$
	"	1	$3.4x10^4$
High-Fives	PlV16tox1	0	$3.8x10^4$
	"	0.001	ND
	"	0.1	$3.9x10^4$
	"	1	$2.9x10^4$
High-Fives	A24toxb4	0	$8.2x10^4$
	"	0.001	$7.1x10^4$
	"	0.1	$2.5 x10^4$
	"	1	$2.5x10^4$
Sf9	PlV16tox1	0	$3.6x10^4$
	"	0.001	$4.3x10^4$
	"	0.1	$7x10^3$
	"	1	$6x10^3$
Sf9	A24toxb4	0	$4.7x10^4$
	"	0.001	$1x10^4$
	"	0.1	$5x10^3$
	"	1	$6.5x10^3$

†ND: cell numbers not determined

For all cell lines, at all protein concentrations tested the maltose binding protein control had no effect on cell growth or viability. Neither of the toxin proteins had any significant effect on cell growth or viability for the *Drosophila melanogaster* Schneider 2 cell line. The *X. nematophilus* A24 toxin caused significant cell growth inhibition and cytotoxicity to the lepidopteran High-Five cell line at concentrations above 0.1μg/ml. The *P. luminescens* V16 toxin caused slight growth inhibition only at the highest concentration tested of 1μg/ml. The *X. nematophilus* A24 toxin caused significant cell growth inhibition and cytotoxicity to the lepidopteran Sf9 cell line at concentrations above 0.001μg/ml, and the *P. luminescens* V16 toxin was toxic to this cell line at concentrations of 0. 1μg/ml and higher. Thus, toxins of this family exhibit growth inhibitory and cytotoxic activity against insect cells in tissue culture, especially cell lines of lepidopteran origin. Similar tests with a mouse hybridoma cell line demonstrated slight growth inhibition only by the *X. nematophilus* A24 toxin, and only at the highest concentration tested of 1μg/ml.

As will be appreciated by persons skilled in this field, the present invention provides a new class of toxins useful for genetically engineering a wide range of biological systems which will thus become more useful for control of pest insects detrimental to agricultural, aquatic and forest industries. This new class of toxin may be purified by one or more methods of protein purification well known in the art. Insecticidal fragments may be generated from the purified toxin using, for example, cleavage with trypsin or cyanogen bromide.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Sequence Listings:

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<150> PO8088

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